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SURVIVAL AND DEVELOPMENT OF TOBACCO HORNWORM LARVAE ON TOBACCO PLANTS GROWN UNDER ELEVATED LEVELS OF OZONE[1]

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Abstract—Tobacco plants, *Nicotiana tabacum* were grown under different levels of ozone (O_3) in open-top chambers. Ozone concentrations were established by charcoal filtration, which reduced O_3 to approximately one-half ambient, or by the addition of O_3 to unfiltered air to increase concentrations to approximately 1.4 or 1.7 times ambient O_3. Survival of tobacco hornworm, *Manduca sexta*, larvae was increased when second instars were fed tobacco leaves grown in chambers with elevated levels of O_3. Second instars also gained significantly more weight when they were fed for one week on plants exposed to elevated levels of O_3 than when they were fed plants grown in charcoal-filtered air. Ozone-treated tobacco plants had higher levels of total nitrogen (primarily reduced nitrogen) and soluble carbohydrates (sugars), and lower levels of leaf-surface components, starch, nicotine, and rutin. Increased survival and growth response of hornworm larvae to elevated O_3 levels in these experiments suggests that similar responses could occur in the

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southeastern US tobacco production areas where O_3 levels can be high enough to injure tobacco plants.

Key Words—Insecta, tobacco hornworm, *Manduca sexta*, tobacco, *Nicotiana tabacum*, ozone, weather fleck, Lepidoptera, Sphingidae.

INTRODUCTION

Tropospheric ozone (O_3) is the most important phytotoxic air pollutant in the United States (Heck et al., 1986; US EPA, 1996). It is produced by reactions requiring sunlight, nitrogen oxides, and volatile organic compounds associated primarily with emissions from motor vehicles. Ozone concentrations in the United States have increased two- to threefold in the last century due to human activity (US EPA, 1996). With average meteorological conditions in the eastern United States during the summer, early morning O_3 concentrations are usually in the 10–30 parts per billion (ppb) range. They increase to 50–100 ppb at peak sunlight and decline toward dusk. Maximum O_3 concentrations vary widely from day to day and may reach 150 ppb several times a year in some areas of the eastern United States (Heagle, 1989). Mean O_3 levels during the daylight hours of the growing season are in the 40–60 ppb range for most agricultural areas of the eastern United States (Heagle, 1989).

Ozone primarily enters plants through open stomata and can cause foliar chlorosis, necrosis, premature senescence, decreased photosynthesis, altered flowering patterns, and reductions in yield (Heck et al., 1988; Heagle et al., 1989). The condition known as weather fleck in tobacco, *Nicotiana tabacum* L., is caused by O_3 (Heggestad and Middleton, 1959), and it may cause significant economic losses (Aycock, 1975; Heagle et al., 1987; Heggestad, 1966).

Insect and mite pests also may be directly or indirectly affected by atmospheric pollutants (Alstad et al., 1982). In some reports, O_3 stimulated insect growth, while in others, O_3 apparently reduced or had no effect on insect development (Heliövaara and Väisänen, 1993). Ozone may change plant resistance or tolerance to insect herbivores, which may exacerbate or offset the direct effects of ozone on the plant (Jones et al., 1994). However, most studies have shown enhanced development rates of chewing insect herbivores that fed on ozonated plants (Heliövaara and Väisänen, 1993). For example, Mexican bean beetles, *Epilachna varivestis* Mulsant, preferentially fed on ozone-exposed soybean foliage over control plants (Endress and Post, 1985). Chappelka et al. (1988) found that Mexican bean beetle larvae also developed faster and weighed more when fed soybeans that had previously been fumigated with O_3. Several studies have reported increased preferences or enhanced survival or development of lepidopteran species on ozonated plants (Bolsinger et al., 1992; Jeffords and Endress, 1984; Trumble et al., 1987). Trumble et al. (1987) reported increased

survival and developmental rates for tomato pinworms, *Keiferia lycopersicella* (Walsingham), that fed on ozonated tomato foliage. Populations of two-spotted spider mites, *Tetranychus urticae* Koch, increased more rapidly on white clover injured by O_3 than on uninjured plants (Heagle et al., 1994).

For the study described herein, we investigated the effects of O_3 and ultraviolet (UV) light on the chemistry and growth of tobacco plants. We also studied how feeding on ozonated tobacco affected the growth and development of tobacco hornworm, *Manduca sexta* L., larvae.

A treatment of enhanced ultraviolet B (UVB) radiation was included in the first experiment, but not in subsequent experiments. The UVB treatment initially was included because of the known deleterious effects that solar UV radiation has on the metabolism, growth, and development of plants (Teramura, 1983). Shorter UVB wavelengths (290–320 nm) can be especially damaging to the photosynthetic system in plants (Berenbaum, 1988; Tevini and Teramura, 1989). In tobacco, the production of certain chemical components can be affected by elevated levels of UVB (Seibert et al., 1975; Barnes et al., 1996). Also, phytophagous insects typically have reduced consumption rates on host plants in the presence of UV radiation (Berenbaum, 1978; Yazawa et al., 1992).

METHODS AND MATERIALS

Tobacco seedlings were started in the greenhouse at the US Department of Agriculture, Agricultural Research Service (USDA-ARS) Crops Research Laboratory, Oxford, North Carolina (this facility was closed in 1994). Plants were grown in cylindrical open-top field chambers (3 m diam. × 2.4 m tall) (Heagle et al., 1973, 1979, 1989) under a range of O_3 concentrations at the USDA-ARS Air Quality Research Unit, North Carolina State University, Raleigh, North Carolina, during 1991 and 1992. For one treatment, air entering chambers was filtered with activated charcoal. The other treatments were unfiltered ambient air, and supplemental O_3 added to unfiltered air to increase concentrations to approximately 1.4 or 1.7 times ambient O_3. Ozone was produced from electrostatic discharge, and levels within all chambers were monitored according to published techniques (Heagle et al., 1979, 1989). Dates, daily hours of exposure, and O_3 concentrations for all experiments are shown in Table 1. Bioassays to measure effects of O_3 stress on growth and survival of hornworm larvae on tobacco leaves were performed in all experiments.

1991 Experiment 1 (Preliminary Experiment). This first experiment must be considered a preliminary test because it had only one open-top chamber for each treatment; and, thus, replication was only within chambers. The four treatments were: charcoal-filtered air with UVB added, charcoal-filtered air with no UVB added, 1.7× ambient O_3 with UVB added, and 1.7× ambient O_3 with no

TABLE 1. DATES, DAILY HOURS OF EXPOSURE, AND CONCENTRATIONS OF OZONE (O_3) FOR EXPERIMENTS TO MEASURE EFFECTS OF O_3 ON GROWTH AND SURVIVAL OF TOBACCO HORNWORMS ON TOBACCO

Year	Experiment	Run	Exposure			Treatment[a]	Mean O_3 conc. (ppb)[b]
			Date begun	Daily duration (hr)	Days		
1991	1	1	May 16	7	64	CF	23
					64	1.7× O_3	90
1991	2	1	Aug. 12	7	24	CF[c]	17
					24	CF–CF[d]	17
					24	1.7× O_3[c]	84
					24	1.7× O_3–CF[d]	65
1991	2	2	Aug. 29	7	21	CF[e]	23
					21	CF–CF[f]	23
					21	1.7× O_3[e]	89
					21	1.7× O_3–CF[f]	67
1992	1	1	June 24	12	35	CF	30
					35	UF	50
					35	1.4× O_3	69
					35	1.7× O_3	83
1992	1	2	Aug. 19	12	34	CF	21
					34	UF	34
					34	1.4× O_3	46
					34	1.7× O_3	57

[a]CF, charcoal-filtered air; UF, unfiltered (ambient) air; 1.4× O_3, supplemental O_3 added to unfiltered air to increase concentrations to approximately 1.4 times ambient; and 1.7× O_3, supplemental O_3 added to unfiltered air to increase concentrations to approximately 1.7 times ambient.
[b]O_3 monitored according to published procedures (Heagle et al., 1979, 1989).
[c]24 days in the same chamber.
[d]17 days in first treatment, then moved for seven days to another CF chamber.
[e]21 days in the same chamber.
[f]14 days in first treatment, then moved for seven days to another CF chamber.

UVB added. The O_3 concentration in the charcoal-filtered air treatments was approximately 20–30 ppb, whereas the O_3 concentration in the 1.7× ambient O_3 treatments was increased for 7 hr per day (14 : 00–21 : 00 hr EDST) to maintain 80–100 ppb O_3. Enhanced UVB was accomplished with banks of lights supplying UVB in the 280–320 nanometer wavelengths (Booker et al., 1992). These lights, which were suspended above the growing plants, raised UVB from an average ambient level of 3.9 kJ/m^2/day to 9.3 kJ/m^2/day (2.4 times ambient). This level was chosen because it has been reported that levels of UVB exposure in this range can detrimentally affect growth, development, and production of chemical components in crop plants (Teramura, 1983; Tevini and Teramura, 1989).

Six plants each of two tobacco entries, NC 2326 and TI 1068, were placed in each chamber. NC 2326 is a flue-cured cultivar that is commonly used as a susceptible check in tobacco insect studies (Jackson et al., 1989). TI 1068 was chosen because it has a different profile of leaf-surface components from commercial tobaccos and is resistant to insect pests (Johnson and Severson, 1982; Severson et al., 1985b). Seedlings were transplanted into 15-liter pots filled with a 2 : 1 : 1 mix of top soil–sand–Metro mix 220 (Scotts Sierra Horticultural Products, Co., Marysville, Ohio). Lime was added to bring the pH to approximately 6.5. Plants were watered daily and fertilized weekly with 10–30–20 (Blossom Booster, Peters). Plants were moved into chambers on May 16, 1991.

Both internal and leaf-surface chemical components were measured from NC 2326 and TI 1068 in each chamber. Samples for the determination of internal chemical components were taken at four, six, and eight weeks after the plants had been put into the chambers. First, the height and number of leaves on each plant were recorded. Each sample for analysis of internal leaf components consisted of four 2-cm-diam. leaf disks taken from the eighth leaf down from the top of four different plants. Three such composite samples were taken for each entry in each treatment chamber. Disks were weighed, placed in scintillation vials containing 10 ml methanol, and frozen on Dry Ice.

Samples for internal leaf chemistry were analyzed for starch, sugar, nitrates, and reduced nitrogen (N). Frozen plant tissues were freeze-dried, weighed, and ground. A portion of each sample was extracted with 80% ethanol to separate soluble carbohydrates and starch. The supernatant was analyzed enzymatically for total sucrose and hexoses (Jones et al., 1977). Total N was determined with an automated CHN analyzer (Perkin-Elmer). Nitrate nitrogen (NO_3) was determined by a modification of the methods of Lowe and Hamilton (1967). Reduced N was determined as total N minus NO_3.

Samples for the determination of leaf-surface chemical components also were taken at four, six, and eight weeks after the plants were put into chambers. Three plants of each tobacco type were sampled within each chamber. These samples were taken from the first leaf below the uppermost leaf showing symptoms of O_3 injury (fifth or sixth leaf down), or the corresponding leaf on the control plants. Samples consisted of five leaf plugs (2 cm diam) each, which were weighed and then dipped into scintillation vials containing 10 ml methylene chloride. These leaf rinses were frozen immediately on Dry Ice and shipped to the Phytochemical Research Unit, Athens, Georgia, for analyses.

Analyses of leaf-surface components included the cembranoid diterpenes, (1*S*,2*E*,4*S*,7*E*,11*E*)-cembra-2,7,11-trien-4-ol (α-CBT-monol) and its (4*R*) epimer (1*S*,2*E*,4*R*,7*E*,11*E*)-cembra-2,7,11-trien-4-ol (β-CBT-monol); the cembranoid diterpenes, (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-cembra-2,7,11-triene-4,6-diol (α-CBT-diol) and its (4*R*) epimer (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-cembra-2,7,11-triene-4,6-diol (β-CBT-diol); the labdanoid diterpenes, (12*Z*)-labda-12,14-diene,8α-ol (*Z*- or

cis-abienol) and (13*E*)-labda-13-ene-8α,15-diol (labdenediol); sucrose esters, 6-*O*-acetyl-3,3,4-tri-*O*-acylsucrose with methyl butyric and methyl valeric acyl moieties; and the predominant fatty alcohol, *N*-docosanol (see Severson et al., 1985b; Wahlberg and Eklund, 1992; and Jackson and Danehower, 1996, for chemical terminology). Labdanoid diterpenes and sucrose esters are found on TI 1068, but not on NC 2326 (Severson et al., 1985b). These analyses were done by published methods (Jackson et al., 1986; Severson et al., 1984, 1985a,b, 1988). Chemical data were analyzed by repeated measures analysis of variance (ANOVA) by the SAS GLM procedure (SAS Institute, 1989). Means of individual components were statistically separated for each sampling date by Duncan's new multiple range test ($P < 0.05$) (SAS Institute, 1989).

On July 12, ten second instars were gently placed by a small paint brush onto the first leaf below the uppermost leaf showing symptoms of O_3 injury (fifth or sixth leaf below the leaf bud), or the corresponding leaf on the control plants of three plants per chamber. Larvae had been reared to second instars on an artificial diet at Oxford, North Carolina (Baumhover, 1985). Larvae were left on the plants for seven days, at which time survivors were counted and weighed. Data for larval survival and weight gain were analyzed by analysis of variance (ANOVA), and means were statistically separated by Duncan's new multiple range test ($P < 0.05$) (SAS Institute, 1989).

1991 Experiment 2. Because in the first experiment UVB at 9.3 $kJ/m^2/day$ had no observable effects on tobacco plant growth or hornworm survival and development, UVB treatments were deleted from further testing. Furthermore, to increase replication, only NC 2326 was used for subsequent experiments. Four treatments were set up in a split-plot design with two replications of each chamber treatment. Ten plants per chamber were grown from August 12 to 28 in chambers with charcoal-filtered air or with 1.7× ambient O_3. Half the plants from each chamber were moved into a separate chamber with charcoal-filtered air on August 28. Then, bioassays were initiated by placing larvae on the first symptomatic leaf below the leaf bud. In this way, we attempted to separate any direct effects that enhanced O_3 might have had on the insects themselves. Thus, the four treatments were: (1) charcoal-filtered air with plants moved to another charcoal-filtered air chamber for bioassays, (2) charcoal-filtered air with plants not moved, (3) 1.7× ambient O_3 with plants moved to a charcoal-filtered air chamber for bioassays, and (4) 1.7× ambient O_3 with plants not moved.

Chemical samples for analysis of leaf-surface components were taken at two and four weeks after the plants had been in the chambers. Leaf disks were weighed and dipped in methylene chloride as described above, and samples were analyzed for cembranoid diterpenes and docosanol at the Phytochemical Research Unit, Athens, Georgia.

On August 29, five second-instar tobacco hornworms were placed on individual symptomatic leaves [the first leaf below the uppermost leaf showing

symptoms of ozone injury (fifth or sixth leaf down), or the corresponding leaf on the control plants] on five plants per treatment. Larvae were left on the plants for seven days, at which time survivors were counted and weighed.

For the second run of experiment 2 in 1991, exposures of new plants to charcoal-filtered air and 1.7× ambient O_3 began on August 29. Half the plants were moved to a new chamber with charcoal-filtered air, and larvae were placed on plants on September 12. Larvae were counted and weighed on September 19. The experimental design was the same as the first run, except infested leaves were enclosed in perforated plastic bags (Del Net; Applied Extrusion Technologies, Inc., Middletown, Delaware) to prevent escape of hornworm larvae and to prevent the influx of native hornworms or predators. The plastic bags had small perforations, which allowed free air exchange, but were too small for the larvae or predators to pass through (Jackson and Severson, 1989). Data from each run were analyzed separately, then combined for ANOVA and means separation by Duncan's new multiple range test ($P < 0.05$) (SAS Institute, 1989).

1992 Experiment. In 1992, two runs of an experiment with NC 2326 were conducted to measure hornworm growth response to plant O_3 stress. The four treatments were: charcoal-filtered air, unfiltered air, 1.4× ambient O_3, and 1.7× ambient O_3. Ozone was added for 12 hr/day (09 : 00–21 : 00 hr EDST) to achieve the 1.4× ambient O_3 and 1.7× ambient O_3 treatments. There were four replications of each treatment (total of 16 chambers). Tobacco transplants were produced in the same fashion as in 1991 to provide 12 plants per chamber. Exposures for the first run began on June 24, and exposures for the second on August 19.

Larvae were placed inside Del Net perforated bags on the leaves at the same stem position (approximately five to six leaves from the apex) in all treatments. Leaves at this position in the charcoal-filtered air treatment were not visibly injured, whereas injury in the other treatments increased as O_3 concentrations increased. Hornworm assays began 28 or 29 days after O_3 exposures began (July 22 and September 14 for runs 1 and 2, respectively). For each run, five second instars were placed in each of five bags per replication. Thus, a total of 100 larvae per treatment were bioassayed for each run. Larvae from both runs were weighed after seven days. Data were analyzed separately for each run, then runs were combined for ANOVA and mean separation by Duncan's new multiple range test ($P < 0.05$) (SAS Institute, 1989).

For each run, chemical samples for analysis of leaf-surface components were taken at two, four, six, and eight weeks after plants had been placed in the chambers. One group of leaf plugs was weighed and dipped in methylene chloride as described above, and samples were analyzed for cembranoid diterpenes and docosanol. A second set of leaf plugs was taken for analyses of internal leaf components. These leaf plugs were weighed, placed in 10 ml methanol, and shipped frozen to the Phytochemical Research Unit, Athens, Geor-

gia, for analyses. These samples were analyzed for alkaloids and polyphenols. Nicotine, the predominant pyridine alkaloid in *N. tabacum*, was analyzed by the GC method of Severson et al. (1981). Polyphenols (chlorogenic acid, rutin, and scopolin) were analyzed directly from the methanol extract by published HPLC methods (Snook and Chortyk, 1982; Snook et al., 1986). Chemical data were analyzed by repeated measures analysis of variance (ANOVA) with SAS GLM procedure (SAS Institute, 1989). Means of individual components at each sampling date were statistically separated by Duncan's new multiple range test ($P < 0.05$) (SAS Institute, 1989).

RESULTS

Chemical Components. Chemical trends were similar for NC 2326 and TI 1068 in the first 1991 experiment (Tables 2 and 3). UVB had little effect on leaf weight, plant height, number of leaves, or levels of chemical components. On the other hand, there were several effects of elevated O_3 including visible leaf injury by four weeks after exposures began. Six weeks after exposures began, plants in the O_3-enhanced treatments had fewer leaves than those grown in charcoal-filtered air, but neither plant height nor leaf density (weight per leaf disk) were affected by any treatment (Tables 2 and 3).

Four weeks after exposure to enhanced O_3 in the first 1991 experiment, both NC 2326 and TI 1068 showed reductions in total starch, and these differences became more dramatic as the experiment progressed (Tables 2 and 3). In contrast, after six weeks (TI 1068) or eight weeks (NC 2326), plants grown under enhanced O_3 had higher levels of soluble sugars.

Levels of reduced N were higher in the enhanced O_3 treatments, and this was significant for both tobacco entries at six weeks (Tables 2 and 3). NO_3, which made up less than 20% of the total N at week 4, dropped to very low levels by the end of this experiment, but the trend for higher levels of NO_3 in the O_3 treatments was apparent at week 6 for NC 2326.

Six weeks (TI 1068) or eight weeks (NC 2326) after O_3 exposures began in the first 1991 experiment, plants in the 1.7× ambient O_3 treatment had decreased levels of cembranoid diterpenes (Tables 2 and 3). For the second experiment in 1991, the reduction in cembranoid diterpenes on NC 2326 was apparent four weeks after treatments began (Table 4). The other leaf-surface components, docosanol (NC 2326), labdanoid diterpenes (TI 1068), and sugar esters (TI 1068), were not affected by enhanced O_3 or UVB in the 1991 experiments (Tables 3 and 4).

Enhanced O_3 treatments also reduced total leaf-surface cembranoid diterpenes by six weeks after exposure in the 1992 experiments (Table 5). The secondary compounds nicotine and rutin also were reduced in treatments with

TABLE 2. CHEMICAL COMPONENTS ON NC 2326 TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS EXPOSED TO DIFFERENT LEVELS OF O_3 AND UVB: EXPERIMENT 1, 1991

Week and treatment	Total cembranoid diterpenes ($\mu g/cm^2$)[a]	Starch (%)[b]	Soluable sugars (%)[b]	Reduced nitrogen (%)[b]	Nitrates (%)[b]	Leaf weight (g)[c]	Plant height (cm)	Leaves (N)
Week 4								
Charcoal-filtered air (CF)	8.0 b[d]	29.6 a	5.0ns[e]	3.9 ns	0.78 ns	0.31 ns	20.3 ns	12.8 ns
O_3-enhanced air (1.7× O_3)	6.8 b	17.9 b	4.7	4.0	0.43	0.33	22.8	11.9
UVB + CF	11.5 a	32.6 a	4.8	3.3	0.23	0.39	20.9	12.3
UVB + 1.7× O_3	8.6 ab	23.8 ab	6.5	3.9	0.22	0.36	22.2	11.6
Week 6								
CF	18.1 ns	53.5 a	5.8ns	2.0 b	0.09 b	0.33 ns	83.7 ns	18.9 a
1.7× O_3	18.1	22.4 b	5.0	3.6 a	0.21 a	0.36	79.4	16.4 b
UVB + CF	18.5	46.9 a	4.8	2.1 b	0.05 b	0.40	79.7	17.7 ab
UVB + 1.7× O_3	17.5	28.8 b	4.1	3.3 a	0.24 a	0.37	76.6	16.2 b
Week 8								
CF	29.3 a	66.6 a	3.1b	1.5 ns	0.012 ns			
1.7× O_3	20.0 b	33.1 b	6.1a	2.2	0.014			
UVB + CF	30.3 a	66.7 a	5.2ab	1.3	0.008			
UVB + 1.7× O_3	24.6 ab	40.6 b	7.2a	1.9	0.018			

[a]Three samples of five leaf plugs each.
[b]Three samples of four leaf plugs each.
[c]Composite weight of four leaf plugs.
[d]For each week, means in the same column followed by the same letter are not significantly different, Duncan's new multiple range test, $P = 0.05$.
[e]Nonsignificant F value in analysis of variance ($P > 0.05$) and no mean separation could be performed.

TABLE 3. CHEMICAL COMPONENTS ON TI 1068 TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS EXPOSED TO DIFFERENT LEVELS OF O_3 AND UVB: EXPERIMENT 1, 1991

Week and treatment	Total cembranoid diterpenes ($\mu g/cm^2$)[a]	Total labdanoid diterpenes ($\mu g/cm^2$)[a]	Sucrose esters ($\mu g/cm^2$)[a]	Starch (%)[b]	Soluable sugars (%)[b]	Reduced nitrogen (%)[b]	Nitrates (%)[b]	Leaf weight (g)[c]	Plant height (cm)	Leaves (*N*)
Week 4										
Charcoal-filtered air (CF)	9.2 ab[d]	1.8 ns[e]	8.5 ab	27.9 a	5.7 ns	4.2 ns	0.95 a	0.36 ns	19.7 ns	12.6 ns
O_3-enhanced air (1.7× O_3)	8.5 ab	1.8	7.4 ab	14.7 b	3.6	5.3	0.41 b	0.32	19.6	12.7
UVB + CF	11.2 a	2.2	9.9 a	33.2 a	5.2	3.3	0.50 b	0.37	18.2	13.2
UVB + 1.7× O_3	7.0 b	1.5	5.2 b	19.3 b	5.7	3.9	0.63 ab	0.32	22.4	12.8
Week 6										
CF	16.6 a	3.1 ns	7.5 ns	53.6 a	3.2 ab	2.8 b	0.11 ns	0.24 ns	64.7 ns	18.4 a
1.7× O_3	12.7 b	3.7	11.3	23.1 b	4.0 a	4.5 a	0.05	0.23	56.3	16.5 b
UVB + CF	15.7 ab	3.4	10.6	49.7 a	2.4 b	2.9 b	0.04	0.25	60.3	17.8 ab
UVB +	13.9 ab	4.2	11.8	30.6 b	3.3 ab	4.8 a	0.18	0.23	65.4	16.2 b
Week 8										
CF	19.3 ns	3.2 ns	8.3 bc	49.5 a	2.5 b	2.6 ns	0.015 ns			
1.7× O_3	18.7	3.1	9.0 b	8.0 b	5.3 a	3.8	0.035			
UVB + CF	19.2	3.7	12.2 a	48.1 a	2.5 b	2.2	0.017			
UVB + 1.7× O_3	20.0	2.9	6.8 c	3.0 b	5.7 a	3.0	0.022			

[a]Three samples of five leaf plugs each.
[b]Three samples of four leaf plugs each.
[c]Composite weight of four leaf plugs.
[d]For each week, means in the same column followed by the same letter are not significantly different, Duncan's new multiple range test, $P = 0.05$.
[e]Nonsignificant F value in analysis of variance ($P > 0.05$) and no mean separation could be performed.

TABLE 4. LEVELS OF SURFACE COMPONENTS AND LEAF WEIGHTS OF NC 2326 TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS UNDER ELEVATED LEVELS OF O_3 OR CHARCOAL-FILTERED AIR: EXPERIMENT 2, 1991

Treatment	Week	Total cembranoid diterpenes ($\mu g/cm^2$)	Docosanol ($\mu g/cm^2$)	Leaf weight (g)[a]
Charcoal-filtered air (CF)	2	4.20	0.10	0.40
Ozone-enhanced air (1.7× O_3)	2	3.50 ns[b]	0.10 ns	0.42 ns
Charcoal-filtered air (CF)	4	11.83	0.15	0.30
Ozone-enhanced air (1.7× O_3)	4	7.45[c]	0.18 ns	0.33 ns

[a]Composite weight of four leaf plugs.
[b]ns, nonsignificant F value in analysis of variance (SAS, 1989).
[c]Significant F value in analysis of variance ($F = 0.0226$).

TABLE 5. CHEMICAL COMPONENTS ON NC 2326 TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS UNDER ELEVATED LEVELS OF OZONE OR WITH CHARCOAL-FILTERED AIR, 1992

Treatment	Week	Total cembranoid diterpenes ($\mu g/cm^2$)	Nicotine ($\mu g/cm^2$)	Total polyphenols ($\mu g/cm^2$)	Rutin ($\mu g/cm^2$)
Charcoal-filtered air (CF)	2	8.0 ns[a]	33.8 a[b]	7.0 ns	1.6 ns
Unfiltered (ambient) air (UF)	2	9.0	30.1 ab	7.2	1.7
Ozone-enhanced air (1.4× O_3)	2	7.9	30.1 ab	7.0	1.5
Ozone-enhanced air (1.7× O_3)	2	7.7	27.4 b	8.1	2.4
CF	4	16.9 ns	56.0 a	13.9 ns	3.0 ns
UF	4	17.7	49.3 a	13.8	3.1
1.4× O_3	4	16.8	49.5 a	14.2	2.9
1.7× O_3	4	14.3	36.8 b	13.5	2.3
CF	6	40.4 a	78.3 a	20.4 ns	4.3 a
UF	6	37.2 a	80.6 a	21.0	4.0 ab
1.4× O_3	6	37.0 a	73.2 ab	19.7	3.6 ab
1.7× O_3	6	30.4 b	55.8 b	19.5	3.2 b
CF	8	27.5 a	74.1 ab	26.1 a	6.8 a
UF	8	28.9 a	81.1 a	25.1 ab	5.8 b
1.4× O_3	8	21.4 b	65.5 ab	24.7 ab	6.0 ab
1.7× O_3	8	22.2 b	51.2 b	22.4 b	4.5 c

[a]Nonsignificant F value in analysis of variance ($P > 0.05$) and no mean separation could be performed.
[b]For each week, means in the same column followed by the same letter are not significantly different, Duncan's new multiple range test, $P = 0.05$.

enhanced O_3 (Table 5). Two other polyphenols, chlorogenic acid and scopolin, were unaffected by enhanced O_3 treatments (data not shown). Significant chlorosis and necrosis of leaves only occurred in the enhanced O_3 treatments. There was no visible injury in the treatment with charcoal-filtered air, and only traces of injury in the unfiltered air treatment. Plants in the treatment with 1.4× ambient O_3 showed minor (less than 10%) leaf injury compared to over 20% injury in the 1.7× ambient O_3 treatment.

The repeated measures ANOVA revealed that all of the chemical data sets had significant repeat effects. However, because we knew in advance that tobacco plants increase production of these chemicals over time as they grow, an effect of repeat was expected. On the other hand, there were no repeat * rep interactions. Only two data sets, sucrose esters (TI 1068 in 1991) and rutin (NC2326 in 1992), had repeat * treatment interactions. For unknown reasons, the sucrose ester data were quite variable, which may have contributed to the significant repeat * treatment interaction (Table 3). There was a low overall response of rutin to the ozone treatments with the effects not being seen until later in the experiment (Table 5).

Survival and Development of Hornworm Larvae. For the first experiment in 1991, hornworm larvae on NC 2326 leaves weighed more in the 1.7× ambient O_3 treatment than in the treatment with charcoal-filtered air (Table 6). However, there were no O_3 weight effects for larvae grown on TI 1068. Enhanced O_3 also

TABLE 6. AVERAGE SURVIVAL AND WEIGHT GAIN OF TOBACCO HORNWORM LARVAE ON TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS EXPOSED TO DIFFERENT LEVELS OF O_3 AND UVB: EXPERIMENT 1, 1991

Tobacco and treatment	Survival (%, mean ± SD)[a]	Average larval weight (g, mean ± SD)[a]
NC 2326		
Charcoal-filtered air (CF)	83.3 ± 5.8 a[b]	0.222 ± 0.023 b
Ozone-enhanced air (1.7× O_3)	76.7 ± 5.8 ab	0.336 ± 0.044 a
CF + UVB	70.0 ± 10.0 ab	0.152 ± 0.023 b
1.7× O_3 + UVB	56.7 ± 15.3 b	0.206 ± 0.057 b
TI 1068		
CF	33.3 ± 32.2 ns[c]	0.139 ± 0.068 ns
1.7× O_3	53.3 ± 28.9	0.126 ± 0.074
CF + UVB	40.0 ± 26.5	0.106 ± 0.062
1.7× O_3 + UVB	60.0 ± 34.6	0.141 ± 0.057

[a]Each value is the mean for three replications (10 larvae per plant, three plants per chamber).
[b]For each tobacco entry, means in the same column followed by the same letter are not significantly different, Duncan's new multiple range test, $P = 0.05$.
[c]Nonsignificant ($P > 0.05$) F value in analysis of variance; therefore, no mean separation could be performed.

TABLE 7. AVERAGE SURVIVAL AND WEIGHT GAIN OF TOBACCO HORNWORM LARVAE ON NC 2326 TOBACCO PLANTS GROWN IN OPEN-TOP FIELD CHAMBERS UNDER ELEVATED LEVELS OF O_3 OR WITH CHARCOAL-FILTERED AIR: EXPERIMENT 2, 1991

Treatment	Survival (%)	Average larval weight (g)[a]	Total larval weight per replication (g)[a]
Charcoal-filtered air (CF), not moved	64.0 ± 9.7 b[b]	0.149 ± 0.027[c]	2.38 ± 1.28 c[b]
CF, moved	69.0 ± 10.5 b	0.182 ± 0.033	3.14 ± 1.40 bc
Ozone-enhanced air (1.7× O_3), not moved	86.0 ± 11.9 a	0.217 ± 0.059	4.67 ± 1.75 a
1.7× O_3, moved	81.0 ± 12.3 a	0.200 ± 0.068	4.05 ± 1.07 ab

[a]Each value is the mean of four replications (five larvae per plant, five plants per chamber; data combined from two runs with two replications each).
[b]Means in the same column followed by the same letter are not significantly different, Duncan's new multiple range test, $P = 0.05$.
[c]Nonsignificant ($P > 0.05$) F value in analysis of variance; therefore, no mean separation could be performed.

had no effect on larval survival for either NC 2326 or TI 1068. Only in the enhanced ozone plus UVB treatment (1.7× ambient O_3 + UVB) for NC 2326 did larvae have reduced survival (Table 6).

For the second experiment in 1991, hornworm survival was greatest in the 1.7× ambient O_3 treatments (Table 7). The composite weights for all surviving hornworm larvae in the 1.7× ambient O_3 treatment were higher than in the charcoal-filtered air. Moving symptomatic plants to another chamber did not affect these results, indicating that there were no direct effects of O_3 on hornworm larvae.

Similar results were obtained in the 1992 experiments (Table 8). Individ-

TABLE 8. AVERAGE SURVIVAL AND WEIGHT GAIN OF TOBACCO HORNWORM LARVAE ON NC 2326 TOBACCO PLANTS GROWN IN OUTDOOR CHAMBERS UNDER ELEVATED LEVELS OF O_3 OR WITH CHARCOAL-FILTERED AIR, 1992

Treatment	Survival (%)	Average larval weight (g)[a]	Total larval weight per replication (g)[a]
Charcoal-filtered air	74.8 ± 11.1 ns[b]	0.296 ± 0.023 b	5.54 ± b
Unfiltered (ambient) air	81.1 ± 12.7	0.308 ± 0.033 ab	6.24 ± ab
Ozone-enhanced air, 1.4× O_3	79.2 ± 16.9	0.307 ± 0.036 ab	6.08 ± ab
Ozone-enhanced air, 1.7× O_3	79.3 ± 11.8	0.339 ± 0.041 a	6.72 ± a

[a]Each value is the mean for eight replications (five larvae per plant, five plants per chamber; data combined from two runs with four replications each).
[b]Nonsignificant ($P > 0.05$) F value in Analysis of Variance; therefore, no mean separations could be performed.

ual weights per larva and total larval weight per replication were higher in the enhanced O_3 treatments for data combined from both runs. For example, larvae from the 1.7× ambient O_3 treatment averaged 0.339 g compared with 0.296 g for larvae from the charcoal-filtered air treatment. However, larval survival was not affected by O_3 in the 1992 experiments.

DISCUSSION

Exposure of plants to subacute levels of O_3 induces many biochemical and physiological changes (Heck et al., 1988; Reich, 1987). There also is much evidence that O_3-induced changes in plants affect insect herbivores (Alstad et al., 1982; Jones et al., 1994; Riemer and Whittaker, 1989; Hughes, 1988; Heliövaara and Väisänen, 1993). This was true in the present study. We have demonstrated physical and chemical changes in tobacco plants induced by elevated levels of O_3, and we have documented increases in the survival and development of *M. sexta* larvae on ozonated plants.

Except for certain Diptera (Beard, 1965; Levy et al., 1972), there is little evidence that atmospheric O_3 directly affects insects (Alstad et al., 1982; Heliövaara and Väisänen, 1993). On the other hand, there is substantial evidence that chronic exposure to O_3 weakens plants or alters them physically or chemically, and, therefore, indirectly affects insects (Alstad et al., 1982). Data from our second 1991 experiment also showed no measurable direct effects of O_3 on hornworm larvae, suggesting that the effects of enhanced O_3 were indirect.

Ozone damage or weather fleck in tobacco has been described physiologically as induced localized senescence and related chlorosis (Heggestad, 1966; Adepipe et al., 1973). Associated with O_3-induced physical damage to tobacco leaves are many changes in levels of chemical components. Menser and Chaplin (1975) reported that field-grown leaves severely damaged by O_3 had increased levels of protein. Aycock (1975) reported that total nitrogen was increased in O_3-damaged leaves. We also observed increases in levels of reduced nitrogen (ca. 90% proteins) in tobacco leaves six weeks after exposure to enhanced O_3. These increased protein levels could enhance growth of tobacco hornworm larvae. However, O_3 exposure also changes the protein pattern in tobacco leaves (Langebartels et al., 1991), including an induction of the defense-related proteins β-1,3-glucanase and chitinase (Schraudner et al., 1992), and it is not clear how these components may affect survival and development of hornworm larvae.

Menser et al. (1977) reported that, in greenhouse experiments, reducing sugars in tobacco leaves were reduced in O_3-polluted air regardless of visible damage. However, Hsieh and Kwan (1972) reported increased levels of sugars four days after ozonation of cigar wrapper tobaccos, but by 15 days after O_3 fumigation, levels of reducing sugars had fallen below those in unfumigated controls.

Kitamura and Kuroda (1973) also reported higher sugars in weather-flecked flue-cured tobacco, but they did not indicate whether leaves were sampled green or cured. We found increased levels of soluble sugars and a corresponding decrease in levels of starch in O_3-enhanced treatments. Increased sugar levels could contribute to increased development by tobacco hornworm larvae.

Trevathan et al. (1979) reported that O_3 fumigation of tobacco leaves resulted in increased lipid concentrations, but decreased concentrations of free sterols and triglyceride fatty acids in all leaf tissues. Menser et al. (1977) found that phytosterols, chlorogenic acid, rutin, scopoletin, and free quinic acid were higher in severely weather-flecked tobacco that had been air-cured. Other researchers have also reported increased polyphenols in O_3-damaged, air-cured tobacco leaves (Aycock, 1975; Menser and Chaplin, 1969). However, these reports differ from our data of slightly lowered levels of rutin in moderately O_3-damaged green tobacco leaves. It is not known whether these differences are attributable to the curing process, varietal differences, or some other factor. Rutin detrimentally affects growth and development of *M. sexta* larvae (Stamp and Skrobola, 1993), so reduced amounts of rutin in ozonated tobacco would favor the increased growth and survival that we observed.

Menser and Chaplin (1969) reported that total alkaloids were reduced in the cured leaves of several tobacco entries severely damaged by weather fleck. Aycock (1975) also reported that alkaloids were reduced in O_3-damaged leaves. Our data agree with these observations.

Although hornworm larvae have adapted to tobacco and are able to excrete nicotine (Self et al., 1964), this process requires energy. Thus, hornworm larvae feeding on tobacco with lowered alkaloid levels would be expected to have increased growth and development. It is likely that both an increase in nutritional factors (e.g., carbohydrates and nitrogenous compounds) and a decrease in toxic secondary components (e.g., polyphenols and alkaloids) in response to increased levels of O_3 contribute to increased survival and weight gain by hornworm larvae. However, we caution that although our data show correlations between levels of chemical components and hornworm development in response to O_3, we have not shown a direct cause-and-effect relationship for any specific leaf component.

Tobacco hornworm moths oviposit 18–41% of their eggs on the middle one third of tobacco plants (Jackson et al., 1991), where the recently mature leaves are found. Thus, many hornworm larvae hatch and initially feed on the leaves most likely to be affected physically and chemically by enhanced O_3. Larvae feeding higher on the plant may be affected by chemical changes in tobacco leaves from enhanced O_3 that take place before physical damage is manifested.

In summary, we have shown that, in general, O_3-treated tobacco plants had higher levels of total N (primarily from reduced N), higher soluble carbohydrates, lower starch, lower levels of certain leaf-surface components (cembranoid

diterpenes), and lower levels of rutin and nicotine. Tobacco hornworm survival and development were increased when they fed on O_3-treated tobacco leaves. Our data suggest that O_3 does not directly affect the growth of hornworm larvae, but instead it has an indirect affect on larvae through changes in the host plant. Increased levels of soluble sugars and protein and reduced levels of nicotine may account for enhanced hornworm growth on leaves showing O_3 damage. Thus, hornworm larvae feeding on plants grown under enhanced O_3 with reduced levels of these toxic components would develop faster and gain more weight. This study gives further evidence that oxidant air pollutants not only affect plants directly, but that they also may increase the detrimental impact of insect herbivores.

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